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Aquimarina litoralis sp. nov., Isolated from a Coastal Seawater

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A strictly aerobic, red-pigmented, non-motile, catalase- and oxidase-positive, Gram-staining-negative bacterium, designated strain CNURIC011^T, was isolated from seawater off the coast of Jeju Island in Korea. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain CNURIC011^T belongs to the genus *Aquimarina* in the family *Flavobacteriaceae*. 16S rRNA gene sequence analysis revealed that the close relatives of the novel strain are *Aquimarina latercula* ATCC 23177^T, *Aquimarina marcrocephali* JAMB N27^T, *Aquimarina intermedia* KMM 6258^T, *Aquimarina muelleri* KMM 6020^T, and *Aquimarina brevivitae* SMK-19^T, with sequence similarities of 97.6, 96.6, 96.0, 95.6, and 94.2%, respectively. DNA-DNA hybridization revealed that the level of relatedness between strain CNURIC011^T and *Aquimarina latercula* ATCC 23177^T (=KCTC 2912^T) was 4.9%. The DNA G+C content was 35.8 mol% and the major respiratory quinone was MK-6. The major fatty acids were iso-$C_{15:0}$ (14.9%), $C_{15:0}$ (13.9%), iso-$C_{17:0}$ 3-OH (12.6%), iso-$C_{15:1}$ G (7.3%), and iso-$C_{17:1}$ ω9c (7.2%). On the basis of phenotypic, phylogenetic, and genotypic data, strain CNURIC011^T represents a novel species within the genus *Aquimarina*, for which the name *Aquimarina litoralis* sp. nov. is proposed. The type strain is CNURIC011^T (=KCTC 22614^T =JCM 15974^T).

***Keywords*:** *Aquimarina litoralis* sp. nov., *Flavobacteriaceae*, taxonomy, seawater

The genus *Aquimarina* belongs to the family *Flavobacteriaceae* and comprises the five species *Aquimarina muelleri*, *Aquimarina latercula*, *Auimarina brevivitae*, *Aquimarina intermedia* (Nedashkovskaya *et al*., 2005, 2006), *Aquimarina marcrocephali* (Miyazaki *et al*., 2009). Nedashkovskaya *et al*. (2005) reported that two of them, *Aquimarina latercula* and *Aquimarina brevivitae* were transferred into the genus *Aquimarina* from *Stanierella* (formerly *Cytophaga*; Lewin, 1969) and *Gaetbulimicrobium* (Yoon *et al.*, 2006), respectively. This genus contains species that are chemoorganotrophic, Gram-staining-negative, strictly aerobic, and in which cytochrome oxidase- and alkaline phosphatase-activities are present. Non-diffusible carotenoid, flexirubin-type pigment production and gliding motility are species-dependent. This study focuses on the taxonomic study of a red-pigmented bacterial strain, designated CNURIC011^T, isolated from the coastal seawater of Jeju Island in Korea. On the basis of taxonomic traits, strain CNURIC011^T represents a novel species within the genus *Aquimarina.*

Materials and Methods

Isolation and culture of bacterial strain

Strain CNURIC 011^T was isolated from a seawater sample collected off the coast of Jeju Island in Korea by using a standard serial dilution plating method and incubation on marine agar (MA) 2216 (Difco, USA) at 25°C for 7 days. Subcultivation was routinely performed on MA at 28°C for 3 days under aerobic conditions and the strain was preserved at -80°C in marine broth (MB, Difco) supplemented with 20% glycerol. This strain was deposited in the Korean Collection for Type Cultures (=KCTC 22614^T) and the Japan Collection of Microorganisms (=JCM 15974^T). For comparative analyses of chemotaxonomic and genetic relationships four types of strains, *Aquimarina latercula* ATCC 23177^T (=KCTC 2912^T), *Aquimarina muelleri* KMM 6020^T (=KCTC 12285^T), *Aquimarina brevivitae* SMK-19^T (=KCTC 12390^T) and *Aquimarina intermedia* KMM 6258T (=JCM 13506^T) were tested with strain CNURIC 011^T.

Determination of 16S rRNA gene sequence, phylogenetic analysis, and DNA-DNA hybridization

Genomic DNA was extracted and purified by using a Wizard genomic DNA Extraction kit (Promega Co., USA), and the nearly complete 16S rRNA gene sequence of strain CNURIC011^T was amplified using two bacterial universal primers, 27F and 1492R (Lane, 1991). The PCR product was purified by using a QIAquick PCR Purification kit (QIAGEN, Germany). Direct sequence determination of the purified 16S rRNA gene was performed with an automated DNA sequencer (ABI 3730XL, Applied Biosystems, USA) at Solgent Co. Ltd (Daejeon, Korea). The resulting 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR) and compared with the available 16S rRNA gene sequences in the GenBank database by using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). The 16S rRNA gene sequences of recognized species in the family *Flavobacteriaceae* were obtained from the GenBank database. Multiple alignments were performed using the CLUSTAL X program (Thompson *et al*., 1997), and gaps at the 5′ and 3′ ends of the

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alignment were edited in the BioEdit program (Hall, 1999). Sequence similarity values were computed using Similarity Matrix version 1.1 (Ribosomal Database Project II; http://rdp.cme.msu.edu/index.jsp; Cole *et al*., 2003) and the EzTaxon server (http://www.eztaxon.org/); Chun *et al*., 2007). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) and maximum-parsimony (Fitch, 1971) in the MEGA 4.0 program (Tamura *et al*., 2007) with bootstrap values based on 1000 replications (Felsenstein, 1985). Evolutionary distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1983). The DNA-DNA hybridization was carried out using the membrane filter hybridization method described by Brown (2005). Probe labelling and hydridization were conducted by using the non-radioactive DIG-High Prime system, and the hybridized DNA was visualized using the DIG luminescent detection kit (Roche, Switzerland) according to the manufacturer's instructions. DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad, USA).

Phenotypic and biochemical characteristics

Colony morphology, size and color were examined from cultures grown aerobically on MA at 28°C for 3 days. The cell morphology and size were examined by light microscopy (Nikon, FDX-35) and transmission electron microscope (JEM-1010, JEOL) using cells from exponentially growing cultures (Bernardet *et al*., 2002; Jeon *et al*., 2004). Flagellar and gliding motility were investigated with exponential phase cells grown on MA at 28°C for 2 days using phase-contrast light microscopy by the hanging drop method as described by Bernardet *et al.* (2002). Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth temperature range was measured from 5 to 45°C (at 5°C intervals) on MA. The pH range for growth was determined in marine broth (MB) 2216 (Difco) adjusted to pH 4.0-11.0 (at intervals of 0.5 pH units) with HCl or NaOH after sterilization. NaCl requirement and tolerance were determined between 0-10% (w/v) NaCl (at 1% intervals) in synthetic Zobell agar medium (ZoBell, 1941; 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, and 15 g Bacto agar in 1 L DW) supplemented with modified artificial seawater (5.94 g $MgSO_4 \cdot 7H_2O$, 4.53 g $MgCl_2 \cdot 6H2O$, 0.64 g KCl, and 1.3 g $CaCl_2$ per L). The production of flexirubin-type pigments was determined by using 20% KOH solution (McCammon and Bowman, 2000; Bernardet *et al.*, 2002). Growth under anaerobic conditions was tested on MA using the GasPak anaerobic system (BBL) at 28°C for 20 days. Catalase activity was evaluated by the production of oxygen bubbles in 3% (v/v) H_2O_2 and oxidase activity was determined by using oxidase reagent (bioMérieux, France). Hydrolysis of Tween 20, Tween 40, Tween 60, Tween 80, casein, starch, and carboxymethyl (CM)-cellulose was investigated on marine agar 2216 after a 7-day incubation at 28°C according to previously described methods (Lanyi, 1987; Tindall *et al*., 2007). Hydrolysis of DNA was tested using DNase test agar (Difco) amended with 3% sea salt. Other phenotypic characteristics and enzyme activity of strain CNURIC011^T were conducted using API 20E, API 20NE, API50CH, and API ZYM test kits (bioMérieux). The use of different carbon sources was tested with the GN2 MicroPlate (Biolog) according to the manufacturer's instructions. Bacterial suspensions were prepared in 3% sea salts. Susceptibility to antibiotics (ampicillin, 10 μg; benzylpenicillin, 10 μg; carbenicillin, 100 μg; erythromycin, 15 μg; gentamycin, 10 μg; kanamycin, 30 μg; lincomycin, 15 μg; neomycin, 30 μg; nalidixic acid, 30 μg; novobiocin, 5 μg; oleandomycin, 15 μg; polymyxin B, 300 μg; tetracycline, 30 μg) was tested on MA plates at 28°C for 3 days.

Chemotaxonomic characteristics

The whole-cell fatty acid composition of strain CNURIC011^T was analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.). Fatty acid methyl esters (FAME) were extracted from cells grown on MA at 28°C for 3 days. The major respiratory quinones were analysed by the Korean Culture Center of Microorganisms (KCCM; South Korea), using reverse-phased HPLC (Komagata and Suzuki, 1987). The G+C content of the DNA was determined by thermal denaturation method (Marmur and Doty, 1962) using Ultrospec 2100 spectrophotometer (Pharmacia Biotech, Sweden). DNA from *Escherichia coli* K-12 was used as a control.

Results and Discussion

Phylogenetic analysis and DNA-DNA hybridization

The nearly complete 16S rRNA gene sequences (1,403 bp) of strain CNURIC011^T was determined and deposited in the GenBank database under the accession number FJ490365. The 16S rRNA gene sequence similarities using the pairwise alignment obtained from the EzTaxon database (Chun *et al.*, 2007) showed that strain CNURIC011^T was closely related to the genus *Aquimarina* in the family *Flavobacteriaceae*. Sequence comparisons with validly published bacteria showed that strain CNURIC011^T shared the highest similarity (97.6%) with *Aquimarina latercula* ATCC 23177^T, followed by *Aquimarina marcrocephali* JAMB N27^T (96.6%), *Aquimarina intermedia* KMM 6258^T (96.0%), *Aquimarina muelleri* KMM 6020^T (95.6%), and *Aquimarina brevivitae* SMK-19^T (94.3%). In the phylogenetic trees based on the neighbor-joining method, strain CNURIC011^T fell within the cluster of the genus *Aquimarina* and formed a distinct phyletic line with type strain of *A. latercula* ATCC 23177^T that is supported by a high bootstrap value of 99% (Fig. 1). Trees based on maximum-parsimony showed similar topology (data not shown). DNA-DNA hybridization was performed to determine genomic relatedness between strain CNURIC011^T and *Aquimarina latercula* KCTC 2912^T (=ATCC 23177^T), which showed the highest level of 16S rRNA gene sequence similarity to the strain. The CNURIC011^T strain evidenced reassociation values of 4.9%. This indicated strongly that the new isolate is a novel species of the genus *Aquimarina* according to the criteria for differentiation of bacterial species (Wayne *et al*., 1987).

Phenotypic and biochemical characteristics

The colonies grown on Marine agar 2216 medium for 3 days at 28°C were circular, convex, smooth, entire, red in color, and 0.5-1.5 mm in diameter. Cells of strain CNURIC011^T were Gram-staining-negative, non-motile, and rod-shaped (0.5 μm in width and 1.0-2.5 μm in length). The strain was able to grow at a temperature range of 10-30°C with an optimum temperature at 25-30°C, but no growth was observed at 4°C or 37°C. The pH and NaCl range for growth was pH 5.5-9.5 (optimum, pH 7.5-8.5) and 1.0-5.0% NaCl (optimum, 3-4% NaCl), respectively. Flexirubin-type pigments were produced. Oxidase and catalase activities were present but nitrate reductase activity was absent. Agar, casein, carboxymethyl (CM)-cellulose, DNA, starch, and Tween 40, Tween 60, and Tween 80 were hydrolysed but Tween 20 and chitin were not. Further descriptions of the strain are given in the species description. Table 1 shows a

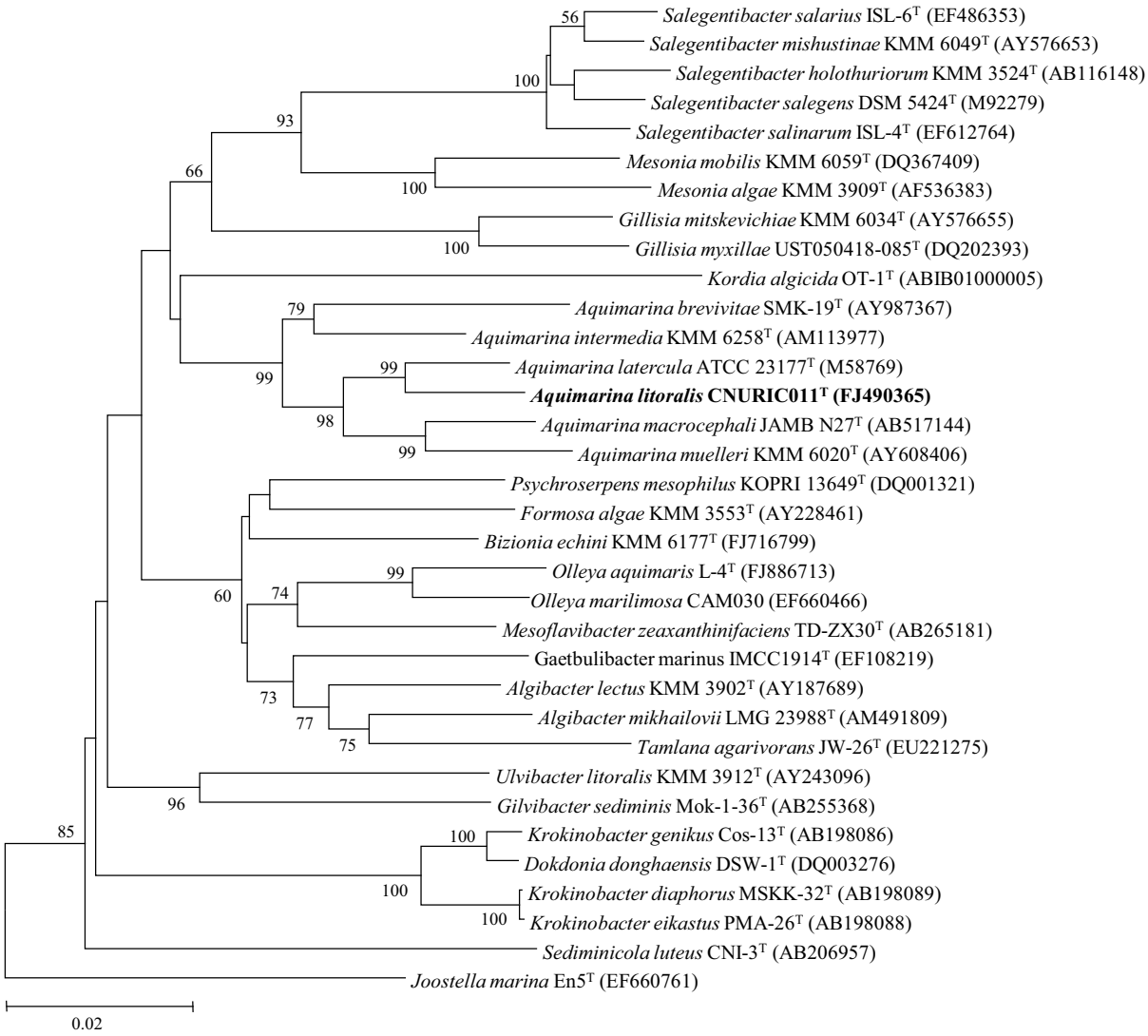

Fig. 1. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequence, showing relationships between strain CNURIC011^T and type strains of species of the genus *Aquimarina*. Bootstrap values are shown in percentages of 1,000 replicates, when greater than 50%. *Bergella zoohelcum* ATCC 43767^T (accession no. M93153) was used as an outgroup (not shown). Bar, 0.02 changes per nucleotide position.

comparison between the characteristics of CNURIC011^T and the other *Aquimarina* type strains.

Chemotaxonomic characteristics

The G+C content of the DNA determined for strain CNURIC011^T was 35.8 mol%, which is within the range of DNA G+C content of the other *Aquimarina* type strains (31-37.1 mol%). The major respiratory quinone was menaquinone-6 (MK-6). The major components of cellular fatty acid profile (>7%) were iso-$C_{15:0}$ (14.9%), $C_{15:0}$ (13.9%), iso-$C_{17:0}$ 3-OH (12.6%), iso-$C_{15:1}$ G (7.3%), and iso-$C_{17:1}$ ω9*c* (7.2%). This fatty acid profile was similar to that of the other *Aquimarina* type strains, although there were differences in the values for some fatty acids (Table 2). The value for $C_{15:0}$ of strain CNURIC011^T (13.9%) was much higher than that of the other *Aquimarina* type strains (1.4-8.2%), whereas the value for iso-$C_{17:0}$ 3-OH of strain CNURIC011^T (12.6%) was less than that of the other *Aquimarina* type strains (16.0-20.9%). The detailed fatty acid compositions of strain CNURIC011^T are given in Table 2.

Taxonomy conclusion

In conclusion, on the basis of the phenotypic characteristics and the phylogenetic analysis results, strain CNURIC011^T represents a novel species of the genus *Aquimarina*, and the name *Aquimarina litoralis* sp. nov. is proposed for this species.

Description of *Aquimarina litoralis* sp. nov.

Aquimarina litoralis (li.to.ra'lis. L. masc. adj. *litoralis*, of the seashore)

Cells are Gram-staining-negative rods, 0.5 μm in diameter and 1.0-2.5 μm in length, strictly aerobic, and non-motile. Colonies on MA are circular, convex, entire, smooth, red-colored, and 0.5-1.5 mm in diameter. Growth occurs at 10-30°C (optimum,

Table 1. Phenotypic characteristics of strain CNURIC011^T and type strain of its closest relatives in the genus *Aquimarina*
Strains: 1, strain CNURIC011^T; 2, *A. latercula* KCTC 2912^T ; 3, *A. intermedia* JCM 13506^T; 4, *A. muelleri* KCTC 12285^T ; 5, *A. brevivitae* KCTC 12390^T; +, positive; –, negative; w, weakly positive. All data were obtained from this study unless otherwise indicated.

Characteristic	1	2	3	4	5
Color of Cell mass	Red	Orange-red	Red	Dark yellow	Orange
Flexirubin production	+	+	+	+	-
Gliding motility	-	-	+	+	+
Growth at/in					
4°C	-	+	+	+	-
37°C	-	+	-	-	+
8% NaCl	-	-	+	+	+
Hydrolysis of					
Agar	+	+	-	-	-
Cellulose	+	+	-	-	-
Chitin	-	+	-	+	-
Starch	+	-	+	+	+
Use of					
L-Arabinose	-	-	+	-	-
D-Glucose	+	-	+	-	-
D-Mannose	+	-	+	-	+
Sucrose	+	-	+	-	-
Susceptible to	+	-	-	+	+
Benzylpenicillin	+	+	+	-	+
Carbenicillin	+	-	+	+	+
Streptomycin	-	+	-	-	+
Tetracycline	+	-	-	+	-
DNA G+C content (mol%)	35.8	34[a]	37.1[a]	31-33[a]	36[a]

[a] DNA G+C content data from Nedashkovskaya *et al*. (2005, 2006) and Yoon *et al*. (2006).

Table 2. Cellular fatty acid compositions (%) of strain CNURIC011^T and type strains of its closest relatives in the genus *Aquimarina*
Strains: 1, strain CNURIC011^T; 2, *A. latercula* KCTC 2912^T; 3, *A. intermedia* JCM 13506^T; 4, *A. muelleri* KCTC 12285^T; 5, *A. brevivitae* KCTC 12390^T. Only the fatty acids amounting to less than 1.0% in all species are not shown. tr, Trace amount (<1%); -, Not detected; ECL, equivalent chain length. All data were obtained from this study.

Fatty acid	1	2	3	4	5
iso-$C_{13:0}$	tr	tr	tr	1.7	tr
iso-$C_{14:0}$	-	tr	-	-	1.1
$C_{15:0}$	13.9	8.2	2.7	1.4	6.6
$C_{15:0}$ 2-OH	1.0	1.0	tr	tr	tr
iso-$C_{15:0}$	14.9	18.9	26.4	26.2	25.7
iso-$C_{15:1}$ G	7.3	5.3	9.8	5.8	12.2
anteiso-$C_{15:0}$	tr	tr	tr	tr	1.0
iso-$C_{15:0}$ 3-OH	5.0	5.0	3.6	6.4	5.2
$C_{16:0}$	4.4	1.7	3.3	3.4	1.0
$C_{16:0}$ 3-OH	tr	1.6	tr	1.9	1.4
iso-$C_{16:0}$	6.4	8.0	2.4	tr	4.6
iso-$C_{16:1}$ H	1.4	1.6	1.3	tr	2.1
iso-$C_{16:0}$ 3-OH	1.0	1.7	1.0	tr	1.6
iso-$C_{17:0}$	1.3	tr	tr	tr	tr
$C_{17:0}$ 3-OH	1.1	1.3	-	-	tr
$C_{17:1}$ ω6*c*	2.7	2.4	tr	-	1.4
$C_{17:1}$ ω8*c*	1.5	tr	tr	-	tr
iso-$C_{17:1}$ ω9*c*	7.2	6.3	11.6	9.2	3.6
iso-$C_{17:0}$ 3-OH	12.6	18.3	18.4	20.9	16.0
$C_{18:0}$	1.3	tr	1.6	1.1	-
Summed features 3[a]	5.7	6.0	5.4	10.1	6.5
ECL 13.565	5.5	4.1	4.2	4.9	3.1

[a] Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 comprises $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH.

25-30°C), at pH 5.5-9.5 (optimum, pH 7.5-8.5), and with 1.0-5.0% NaCl (optimum, 3.0-4.0%). Catalase and oxidase activities are present. Flexirubin-type pigments are produced. Agar, casein, starch, carboxymethyl(CM)-cellulose, DNA, Tween 40, Tween 60, and Tween 80 are hydrolysed, but Tween 20 and chitin are not. In API 20E tests, production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, and tryptophan deaminase, use of citrate and Voges-Proskauer reaction are negative. In API 20NE tests, nitrate is reduced to nitrite, but indole production, urease activity, glucose fermentation and gelatin hydrolysis are negative. In the API ZYM tests, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, and β-galactosidase activities are present. Esterase lipase (C4), lipase (C14), cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities are absent. Does not produce acid from carbohydrate. Oxidizes the following carbon substrates (Biolog GN2 microplates): α-cyclodextrin, dextrin, glycogen, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, m-inositol, α-D-lactose, lactulose, maltose, D-mannose, β-methyl-D-glucoside, D-sorbitol, sucrose, D-trehalose, turanose, acetic acid, *cis*-aconitic acid, citric acid, D-galacturonic acid, D-glucosaminic acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, glucuronamide, L-alanine, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-histidine, hydroxy-L-proline, L-pyroglutamic acid, L-serine, D-serine, γ-amino butyric acid, inosine, D-L-α-glycerol phosphate, α-D-glucose-1-phosphate, and D-glucose-6-phosphate. All of the other carbon sources not listed here in the GN2 system are not utilized. Susceptible to ampicillin (10 μg), benzylpenicillin (10 μg), carbenicillin (100 μg), erythromycin (15 μg), lincomycin (15 μg), nalidixic acid (30 μg), novobiocin (5 μg), oleandomycin (15 μg), and tetracycline (30 μg), but resistant to gentamycin (10 μg), neomycin (30 μg), polymyxin B (300 μg), kanamycin (30 μg), and streptomycin (10 μg). The major cellular fatty acids are iso-$C_{15:0}$ (14.9%), $C_{15:0}$ (13.9%), iso-$C_{17:0}$ 3-OH (12.6%), iso-$C_{15:1}$ G (7.3%), and iso-$C_{17:1}$ ω9*c* (7.2%). The DNA G+C content of the type strain is 35.8 mol% and the major respiratory quinone was menaquinone-6.

The type strain, CNURIC011^T (=KCTC 22614^T =JCM 15974^T), was isolated from seawater off the coast of Jeju Island in Korea.

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